No increase in C-reactive protein levels during intranasal compared to oral hormone therapy in healthy post-menopausal women

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BACKGROUND: Inflammation plays an important role in the development of atherosclerotic disease. Oral post-menopausal hormone therapy increases serum C-reactive protein (CRP) levels. This study compared the effects of intranasal and oral administration of 17β-estradiol (E₂) combined with norethisterone acetate (NETA) on markers of inflammation in healthy post-menopausal women. METHODS: Ninety healthy post-menopausal women (age 56.6 ± 4.7 years) participated in this 1-year trial. After computerized block randomization, they daily received, in a double-blind fashion, either intranasal E₂/NET [175 µg/275 µg (n = 47)] or oral E₂/NETA [1 mg/0.5 mg (n = 43)]. Concentrations of high sensitivity CRP and adhesion molecules were measured at baseline and after 12, 24 and 52 weeks of treatment. RESULTS: CRP levels were increased (P = 0.001) in the oral but not in the intranasal group. The increase in the oral group was highest at week 12 (64.9%) and was larger (P < 0.01) compared with the non-significant increase (8.6%) found in the intranasal group. Both groups showed decreases (P < 0.001) in soluble vascular cell adhesion molecule (sVCAM), soluble intracellular adhesion molecule (sICAM) and sE-selectin. The decreases were larger (P < 0.01) in the oral than in the intranasal group. CONCLUSION: Intranasal E₂/NET therapy did not significantly increase CRP levels, in contrast to the increase observed in the oral E₂/NETA treatment group. Both intranasal and oral therapy lowered plasma concentrations of adhesion molecules, however, more so in the oral group.

Key words: adhesion molecules/C-reactive protein/intranasal/ menopause/ norethisterone

Introduction

The evidence available as to the effect of post-menopausal hormone therapy (HT) on cardiovascular risk is conflicting. In contrast to observational studies reporting a risk reduction among HT users (Barrett-Connor and Grady, 1998), randomized controlled trials reported no effect or even an early increased risk for the development of coronary heart disease (CHD) among women assigned to oral HT (Hulley et al., 1998; Manson et al., 2003; Anderson et al., 2004).

Inflammation plays an important role in the process of atherosclerosis and of plaque formation (Ross, 1999). Acute phase proteins, especially C-reactive protein (CRP), play a role in non-specific defence mechanisms. High sensitivity CRP (hsCRP) has been shown to be a predictor of future cardiovascular events (Ridker et al., 1997; Ridker et al., 2000). In response to endothelial dysfunction, cell adhesion molecules are expressed on endothelial cells to attract leukocytes. Elevated plasma levels of these molecules have been associated with an increased CHD risk (Hwang et al., 1997; Ridker et al., 1998). Most controlled studies report an increase in CRP levels during oral HT (Van Baal et al., 1999; Post et al., 2002; Skouby et al., 2002; Lamon-Fava et al., 2003; Bukowska et al., 2005). During transdermal HT, the liver, the major production site of CRP, is bypassed. Most studies found no increase during transdermal HT (Vehkavaara et al., 2001; Post et al., 2002; Lacut et al., 2003; Vongpatanasin et al., 2003; Yilmazer et al., 2003; Bukowska et al., 2005). For adhesion molecules, both oral and transdermal HT studies have shown inconsistent results (Seljeflot et al., 2000; Vehkavaara et al., 2001; Hemelaar et al., 2005).

In addition to the oral and transdermal route of administration, a spray has become available for intranasal administration of estradiol (E₂) (Aerodiol®, Servier, Courbevoie, France). The intranasal route has been shown to be a well-tolerated, effective alternative (Studd et al., 1999; Lopes et al., 2000; Mattsson et al., 2000). Less intra- and inter-subject variability was observed in E₂ exposure (Studd et al., 1999; Lopes et al., 2000). As a successor to the E₂-only spray, an intranasal spray for continuous combined 17β-E₂ and norethisterone (NET)
administration has been developed. As hepatic metabolism is largely bypassed, it is plausible that, just as transdermal patches or gels and the intranasal E₂-only spray (Kiran et al., 2004) have little effect on CRP levels, the same may be true of the intranasal E₂/NET combined spray. In the present study, the effects on CRP and adhesion molecules of the intranasal E₂/NET formulation were compared with those of oral low-dose continuous combined E₂/norethisterone acetate (NETA), a widely marketed combination. Nestled within a large international, randomized, double-blind, double-dummy study, which had endometrial safety as the primary endpoint, effects on CRP and adhesion molecules were studied as a secondary objective among participants in two Dutch centres.

Subjects and methods

Participants

Healthy post-menopausal women, aged 40–75 years, were recruited from outpatient clinics and through advertisements in regional newspapers. All the women were non-hysterectomized and had their last menstrual period at least 2 years before inclusion. Serum E₂ had to be <30 pg/ml with FSH >30 mIU/ml. All participants had a normal cervical smear and mammography within 12 months before inclusion. A normal transvaginal ultrasound and an endometrial biopsy without hyperplasia or polyps were required, also blood tests (lipids, liver enzymes, kidney function, glucose and thyroid-stimulating hormone) without any clinically relevant abnormalities. At screening, all participants had plasma levels of total cholesterol of ≤8.0 mmol/l and of triglycerides of ≤3.0 mmol/l. Exclusion criteria were a BMI >32 kg/m², any contraindication to the use of estrogen and/or progestogen, any ear–nose–throat disease that might interfere with intranasal drug administration and concomitant use of the following treatments: any treatment for menopausal symptoms, chronic treatment liable to interfere with the coagulation profile, treatment liable to interfere with intranasal administration, enzyme inducers and systemic vasoconstrictors. Inflammation parameters were measured in a subset of women who either had no history of use of HT or had a washout of previous HT of at least 6 weeks before the baseline visit and who were not taking lipid-lowering drugs.

All participants gave written informed consent before inclusion in the trial that was conducted in accordance with the ethical principles stated in the Declaration of Helsinki, with Good Clinical Practice and with approval of the central and local institutional review boards.

Study design

This study was performed in 90 women who were included in two centres in the Netherlands, as part of a large international, randomized, double-blind, double-dummy study with two parallel treatment arms including 954 women in total. After a 1–6 weeks screening period, eligible women were randomized to either one intranasal spray containing a fixed dose of 175 µg 17β-E₂ + 275 µg NET (S21405, Servier, Courbevoie, France) and one placebo capsule (intranasal E₂/NET group) or one capsule containing 1 mg 17β-E₂ + 0.5 mg NETA (Activelle® Novo Nordisk, Bagsvaerd, Denmark) and one placebo intranasal spray (oral E₂/NETA group) daily. Study medication was manufactured, packaged and labelled by the Institut de Recherches Internationales Servier (I.R.I.S.; Courbevoie, France). Placebos and active treatments were identical in appearance and smell. Centralized computerized subject randomization was done by an Interactive Voice Response System (I.V.R.S.) in blocks of 12 (six active spray and six active capsules) per centre. Treatment was administered for 52 weeks.

Throughout the whole study period, all participants, clinical investigators and laboratory personnel were blinded for the study medication. Unblinding was done after all data were collected in the database.

Markers of inflammation

To assess concentrations of CRP and adhesion molecules, venous blood samples were taken at baseline and at weeks 12, 24 and 52. Blood samples were taken between 8.00 and 10.00 a.m. after fasting and non-smoking for at least 10 h and no alcohol intake for at least 24 h. After 20 min of rest, blood was collected into plain tubes at room temperature (Becton Dickinson, Plymouth, UK) for CRP or into pre-cooled tubes containing K3-EDTA for soluble vascular cell adhesion molecule (sVCAM), soluble intracellular adhesion molecule (sICAM) and sE-selectin. After blood collection, pre-cooled tubes were immediately put back into ice. Within 1 h after collection, plasma was separated by centrifugation at 2000 g for 30 min at 20°C (for blood collected into plain tubes) or at 4°C (for blood collected into cooled tubes containing K3-EDTA). Plasma was divided into aliquots, snap-frozen and stored at −80°C until analysis.

hsCRP was assayed by an in-house sensitive ELISA with a lower limit of detection of 0.01 mg/l. Adhesion molecules were determined by commercially available ELISAs (Elipair, Diaclene, Besançon cedex, France). The intra- and inter-assay coefficients of variation (CVs) were 3.9 and 8.7% for CRP, 4.4 and 5.1% for sVCAM, 4.0 and 6.4% for sICAM and 4 and 10% for sE-selectin, respectively. All samples of a given subject were assayed within a single run.

Plasma levels of E₂ and of FSH were measured on the Roche E-170 Modular (Roche, Basel, Switzerland) using electrochemoluminiscence immunoassay (ECLA), serum sex hormone-binding globulin (SHBG) levels were measured using an immunoradiometric assay (IRMA) (Orion Diagnostica, Espoo, Finland), which has an overall CV of 10%.

Statistical analyses

Statistical analyses were performed using the Statistics Package for Social Sciences (SPSS) 11.5 for Windows (SPSS Inc, Chicago, IL, USA). Concentrations of the inflammation parameters are given as mean ± SD or as median (25th–75th percentile) if skewed. Percentage changes from baseline are given as mean 95% confidence interval (CI) or as geometric mean (95% CI) if the changes had a skewed distribution.

Statistical analyses were performed using standard parametric tests; if the variables were skewed, analyses were done after log-transformation. Baseline values were compared using an unpaired t-test or a Chi-square test where applicable. Within-group changes over time were tested using analysis of variance (ANOVA) for repeated measurements and paired t-tests versus baseline. For between-group comparisons, we used analyses of covariance (ANCOVA) for repeated measurements, with the baseline value of the variable under consideration as constant covariate. Percentage changes from baseline were compared with unpaired t-tests.

Only data from women for whom data were available at baseline and at least at one other time point were used for the analyses; for ANOVA and ANCOVA for repeated measurements, the last-observation-carried-forward procedure for the missing values was applied.

As CRP levels were raised by inflammatory conditions, we re-analysed CRP data after exclusion of women (n = 10) who reported an infection in the weeks before blood sampling or those with a CRP concentration ≥10 mg/l, which is considered as the lower threshold level for the existence of an acute inflammation.

Correlations for baseline values and absolute changes in CRP and adhesion molecules were analysed by calculation of the Pearson’s
correlation coefficient. Correlations with changes in SHBG plasma levels were analysed in the subset of women whose SHBG values were available \((n = 65, 54 \text{ and } 51 \text{ at weeks } 12, 24 \text{ and } 52, \text{ respectively})\).

Sample size calculation for this cardiovascular substudy was based on changes in normalized APC sensitivity ratio (nAPCsr), a strong haemostatic risk factor associated with an increased venous thrombotic risk, and is the pathophysiologic mechanism behind the factor V Leiden mutation, a hereditary risk factor for venous thrombosis. To find a 35% difference in change in nAPCsr between the groups with a SD in percentage change of 75%, using a power of 80% and an alpha group.

botic risk, and is the pathophysiologic mechanism behind the factor V because of the start of prevent anticoagulant therapy on the detection of breast cancer at week 38, and one woman in Furthermore, one woman in the oral group stopped because of which, however, could not be confirmed ultrasonographically. 

In retrospect, probably was already present before study entry. 

Because of the occurrence of a deep venous thrombosis which, 

Furthermore, in the current study, fewer women in the intranasal group.

Overall, we found significant \((P < 0.001)\) reductions in both groups in sVCAM, sICAM and sE-selectin (Figure 2b). These were larger in the oral compared with the intranasal group \((P < 0.01 \text{ for } sVCAM \text{ and } sICAM \text{ and } P < 0.001 \text{ for } sE-selectin)\). After 12 weeks of treatment, the reductions in the intranasal and in the oral group were –9.6 and –14.6% in sVCAM, –9.5 and –14.5% in sICAM and –8.8 and –12.3% in sE-selectin, respectively \((\text{all } P < 0.001 \text{ versus baseline})\). Reductions persisted until week 52.

At baseline, there was a significant correlation between CRP and BMI \((r = 0.29, P < 0.01)\) and between values of sE-selectin and sVCAM \((r = 0.30, P < 0.01)\). There were no other significant correlations among the baseline values of CRP, sVCAM, sICAM and sE-selectin. Changes in sVCAM and sICAM and in sVCAM and sE-selectin were positively correlated at week 12 \((r = 0.45, P < 0.001 \text{ and } r = 0.36, P = 0.001, \text{ respectively})\) and this persisted at week 24 and 52. Changes in CRP did not correlate with changes in adhesion molecules or with changes in SHBG.

Re-analysis after exclusion of those women who reported an infection in the weeks before blood sampling or with a CRP concentration ≥10 mg/l (intranasal \(n = 4, \text{ oral } n = 6)\) revealed highly similar results (data not shown).

Smokers had significantly \((P < 0.05)\) higher baseline values of sICAM than non-smokers. Between-group differences in changes in CRP and adhesion molecules remained highly similar when smoking was added as an extra covariate in ANCOVA. Changes within the treatment groups did not differ between smokers and non-smokers (data not shown).

Discussion

In this study, we investigated the effect of intranasal administration of combined estrogen plus progestogen therapy on various markers of inflammation, which are indicators of atherosclerosis. Intranasally administered E2 is rapidly absorbed and induces very steep and short peaks in serum levels. This pulsed exposure affects the sensitivity of some target tissues to estrogen (Kenemans et al., 2004). In endothelial cells, a similar effect was observed after pulsed E2 exposure to that found after continuous exposure. However, it appeared to be induced through non-genomic pathways rather than the genomic pathways during continuous exposure (Simoncini et al., 2005). An important potential advantage of the difference in mechanism might be the low stimulation of the breast and endometrium (Kenemans et al., 2004). In the current study (Hemelaar et al., in press), as well as in a previous study (Mattsson et al., 2000), fewer women \((P < 0.01)\) reported breakthrough bleeding and mastalgia in the intranasal group. Furthermore, in the current study, fewer women in the intranasal group, compared with the oral group, discontinued the study because of adverse events.

Improvement of climacteric complaints and 24 h exposure to E2 after intranasal administration of 300 µg E2 was shown to be similar when compared with oral 2 mg E2 (Devissaguet et al., 1999; Mattsson et al., 2000) and transdermal 50 µg E2 (Devissaguet et al., 1999; Lopes et al., 2000). Bioavailability of E2 was more stable after intranasal than after oral administration.

Results

Participants

Between September 2001 and June 2002, 125 women were screened in two participating centres, of whom 94 women were randomized. As four women either had no washout from previous HT \((n = 3)\) or used lipid-lowering drugs \((n = 1)\), 90 women were found to be eligible for the current substudy (Figure 1). The last group of patients completed the study in May 2003.

Baseline demographic characteristics did not differ between the two treatment groups (Table I).

Two women in the intranasal group discontinued the study compared with 10 in the oral group \((P < 0.01)\) (Figure 1). Premature study discontinuation was mainly related to the occurrence of an adverse event. No women stopped because of the occurrence of a coronary or a cerebrovascular event. One woman in the intranasal group discontinued at week 11 because of the occurrence of a deep venous thrombosis which, in retrospect, probably was already present before study entry. Another woman, in the oral group, discontinued at week 36 because of clinical symptoms fitting a venous thrombosis which, however, could not be confirmed ultrasonographically. Furthermore, one woman in the oral group stopped because of the detection of breast cancer at week 38, and one woman in the intranasal group was excluded from analyses after week 12 because of the start of preventive anticoagulant therapy on account of a family history of cerebrovascular disease.

Analyses were therefore based on 86 women (46 in the intranasal and 40 in the oral group) for whom values at baseline and at least at one other time point were available.

Markers of inflammation

Baseline values of CRP and adhesion molecules were similar between the groups (Table II).

During the 52-week study period, CRP levels increased significantly \((P = 0.001)\) in the oral but not in the intranasal group (Figure 2a). The difference between the groups was statistically significant \((P = 0.01)\). At week 12 and 24, the increases from baseline in the oral group were 64.9% \((P = 0.001)\) and 49.1% \((P < 0.01)\), respectively, whereas non-significant increases of 8.6 and 29.8% were found in the intranasal group. The difference between the groups at week 12 was statistically significant \((P < 0.05)\). At week 52, the increase from baseline in both groups was non-significant \((19.8% \text{ in the oral and } 4.2\% \text{ in the intranasal group})\).
M. Hemelaar et al. 1638

NETA is rapidly hydrolysed into the active hormone NET, and equivalent quantities of NETA and NET provide similar pharmacokinetic profiles for NET concentrations (unpublished data). Therefore, NET was used in the intranasal spray.

In the oral E₂/NETA group, we found a significant 64% increase in CRP and significant decreases in sICAM, sVCAM and sE-selectin. During oral HT, most placebo-controlled studies report an increase in CRP (Cushman et al., 1999; Van Baal et al., 1999; Post et al., 2002; Skouby et al., 2002; Lamon-Fava et al., 2003; Bukowska et al., 2005) and a decrease in adhesion molecules (Cushman et al., 1999; Guzic-Salobir et al., 2001; Hemelaar et al., 2005). An increase in CRP has been found to occur within 2 weeks after start of oral HT (Ropponen et al., 2005). The magnitude of the increase in CRP appears to be dose related (Koh et al., 2004; Prestwood et al., 2004; Wakatsuki et al., 2005).

Figure 1. Trial profile. n, number of subjects; Intranasal, spray containing 175 μg estradiol (E₂) and 275 μg norethisterone; oral, capsule containing 1 mg E₂ and 0.5 mg norethisterone acetate.
et al, 2004; Ropponen et al, 2005) and even a decrease during ultra low-dose E2 (0.25 mg) has been described (Prestwood et al, 2004). Addition of a progestogen can attenuate the increase in CRP (Van Baal et al, 1999; Post et al, 2002), depending on the type of progestogen used (Skouby et al, 2002).

In the intranasal E2/NET group, we are the first to report no significant increase in CRP and significant decreases in soluble adhesion molecules. Until now, only one study has reported the effects of intranasal estrogen therapy on inflammation parameters (Kiran et al, 2004), finding no increase in CRP after 6 months of unopposed intranasal E2. As to other non-oral regimens, transdermal HT is most widely studied. Compared with placebo, most studies reported changes in CRP during various transdermal E2 regimens to be non-significant in healthy postmenopausal women (Vehkavaara et al, 2001; Post et al, 2002; LaCut et al, 2003; Vongpatanasin et al, 2003; Yilmazer et al, 2003; Bukowska et al, 2005). However, one study in women with type 2 diabetes reported a decrease in CRP during transdermal E2 plus oral NETA (Sattar et al, 1999). In general, adhesion molecule levels were reported to be decreased (Seljeft et al, 2000) or unchanged (Vehkavaara et al, 2001; Hemelaar et al, 2005).

The increase in CRP as well as the decrease in adhesion molecule levels was larger in the oral than in the intranasal group. This was also found in studies making a direct comparison between oral and transdermal regimens (Post et al, 2002; LaCut et al, 2003; Strandberg et al, 2003; Bukowska et al, 2005), including one study comparing oral and transdermal E2/NETA (Strandberg et al, 2003).

Because cigarette smoking is an important cause of endothelial dysfunction (Ross, 1999), the effects of smoking were evaluated in this study. The difference in sICAM at baseline between smokers and non-smokers did not influence the results, as smokers were equally divided among the oral and the intranasal groups. The finding that changes in CRP and in adhesion molecules were not affected by smoking status has been reported earlier (Cushman et al, 1999).

It has yet to be determined what this increase in CRP levels during oral HT reflects. CRP appears to have a dualistic role in inflammation. On the one hand, it is a non-specific marker of inflammation, with hsCRP reflecting the amount of infiltrate in atherosclerotic plaques. On the other hand, CRP appears to be able to aggravate an inflammatory response in the vascular wall by itself; exposure to CRP has been shown to induce expression of adhesion molecules on human endothelial cells (Pasceri et al, 2000), and a higher rate of thrombotic occlusion was observed in the presence, rather than in the absence, of CRP in a mouse model (Danenberg et al, 2003). CRP appears to contribute to inflammation through complement activation.
as was seen in ischaemic myocardium after infarction (Lagrand et al., 1997) and in early atherosclerotic coronary arteries (Torzewski et al., 1998). In both scenarios, the increase in CRP levels is an unwanted effect of oral HT, which can be avoided by administration by the non-oral route.

The absence of an increase in interleukin-6 (IL-6) and tumour necrosis factor α (TNFα) (Lacut et al., 2003; Vongpatanasin et al., 2003), both important promoters of CRP synthesis in response to inflammation, provides evidence that counters the hypothesis that HT directly induces an inflammatory state. Furthermore, in vitro and animal studies have not demonstrated any pro-inflammatory response to HT, and the reduction in adhesion molecule levels during oral HT suggests an anti-inflammatory effect. Also, when similar increases in plasma E2 levels were obtained during oral and transdermal HT, CRP levels were raised during oral but not so during transdermal treatment (Vongpatanasin et al., 2003). An increase in plasma SHBG levels during oral HT is regarded as an indicator for estrogenic effects on hepatic protein synthesis. The absence of a correlation between the change in CRP and in SHBG which we found has been described before (Ylikorkala et al., 2003) and argues against the suggestion that the increase in CRP is just a hepatic first-pass effect.

Hepatic production of a pro-inflammatory factor during oral HT plays a role in an interesting hypothesis to unify the apparently cardioprotective effect of HT in observational studies and in an increased risk of CHD during the first year of HT in predominately late post-menopausal women in the Heart and Estrogen/progestin Replacement Study (HERS) (Hulley et al., 1998) and in the Women’s Health Initiative trial (WHI) (Manson et al., 2003). In late post-menopausal women, who most likely have more extensive vulnerable plaques, hepatic production of prothrombotic and pro-inflammatory factors may outweigh potentially beneficial effects on lipids and anticoagulant factors, therefore inducing plaque instability (Phillips and Langer, 2005). However, in early post-menopausal women, in whom less vulnerable plaques are present, decreased plaque production after start of HT leads to a decrease in CHD (Phillips and Langer, 2005). The temporary increase in CRP levels we found in the oral group during the first 24 weeks has been described previously (Yilmazer et al., 2003). We can only speculate about the mechanisms of this later decrease in CRP levels. Among the possibilities is the later onset of CRP-lowering mechanisms such as the following: (i) clearance, in association with the increased clearance of low-density lipoprotein (LDL) with which CRP appears to interact closely (Ji et al., 2005); and (ii) inhibition of protein production in general, as reflected in lower albumin levels (Smolders et al., 2005), or a decrease in CRP-levels due to raised SHBG-levels (Sowers et al., 2005). This temporary effect may account for the early CHD risk increase reported in the large randomized controlled trials (Hulley et al., 1998; Manson et al., 2003).

One of the strengths of our study is the randomized, double-blind study design and the 1-year duration including the measurement of short-term effects after 3-months therapy. The study also has limitations. We did not include an untreated or placebo-controlled group. However, we compared the effects of the new intranasal spray with a reference product, the effects of which were found to be highly similar to those observed in placebo-controlled studies.

In conclusion, in this study in healthy post-menopausal women, intranasal continuous combined E2/NET therapy did not significantly increase CRP levels, in contrast to the increase observed in the oral group within the first year of use. Both intranasal and oral therapy lowered adhesion molecules; however, the effects were more pronounced in the oral group. These findings suggest a favourable effect of intranasal post-menopausal HT compared to oral therapy.

Acknowledgements

We thank all women who participated in the study; C.Klipping, MD, Dinox Medical Investigations, Nijmegen, M.O.Verhoeven, MD and T.E.Vogelvang, MD, PhD, VU University Medical Center, Amsterdam, for logistical and technical assistance; A.Kok, Department of Clinical Chemistry, VU University Medical Center, Amsterdam, for laboratory assistance. This study was supported by a research grant from the Institut de Recherches Internationales Servier (I.R.I.S., Courbevoie, France) to the Biocare Foundation (grant no 01-049).
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Submitted on November 14, 2005; resubmitted on January 17, 2006; accepted
on January 19, 2006